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ENVIRONMENTAL RESEARCH

DEVELOPMENT OF DNA PROBE(S) FOR THE DETECTION OF *Bifidobacterium Spp.* IN WATER

FINAL REPORT

RAC Project No. 629C

MINISTRY OF ENVIRONMENT AND ENERGY

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EXECUTIVE SUMMARY

In earlier studies (Phases I and II) DNA inserts of several *B. adolescentis* recombinant clones have been found, after elaborate hybridized analyze, to show some specificity to *Bifidobacterium* species. Clones pA93 and p83 hybridized to DNA of *B. adolescentis* and the human *Bifidobacterium* isolate 'MEB'. Clones pA34 and p63 are more specific to *Bifidobacterium* as these two clones hybridized to DNA of all three *Bifidobacterium* species tested.

Further characterization revealed that radioactively-labelled pA34 probe hybridized strongly to DNA of *B. adolescentis*, *B. longum, B. brave* (two different strains) and *B. bifidum*. It was weakly hybridized to *E. coli* and *Shigella soneii*, and did not hybridized to *Lactobacillus caseii, Campylobacter jejuni* and *Pseudomonas aeruginosa*. The radioactive pA34 probe could detect as low as 10 ng DNA of *B. adolescentis* and a minimum of 2 x 10^5 cells of *B. adolescentis*.

The insert in pA34 was sequenced and it showed 65% homology to a part of the L5 ribosomal protein gene of *E. coli*. The sequence information enabled the selection of two conserved regions, using CLUSTAL V database program. These conserved regions amplified a 288 bp in five *Bifidobacterium* species, *B. adolescentis*, *B. bifidum*, *B. brave*, *B. infantis* and *B. langum*. Based on sequence analysis, two PCR primers, PFL5-1 and PFL5-2 and a *Bifidobacterium* genus-specific probe, BFP23, were designed.

This study provided that BFL5-1 and BFL5-2 primers can amplify specific segment of L5 ribosomal protein gene in the five chosen *Bifidobacterium* (*B. aldolescent, B. bifidium, B. breve, B. infantis,* and *B. longum*). The BFP23 probe hybridized strongly to all five chosen *Bifidobacterium* at 62°C, weakly to *P. aeroginosa*, and negative to the other six targeted bacteria. The detection limit of this assay is 20 copies of the targeted gene. The detection assay can be improved by decreasing the degeneracy of the PCR primers and design an oligonucleotide probe in another conserved region of the L5 gene sequence of the five *Bifidobacterium* species.

INTRODUCTION

Although the Ontario Ministry of Environment has been putting in extensive efforts in monitoring water quality by means of identifying and eliminating sources of faecal pollution in waters, no standard protocol has been systemized for all environment, especially when specific bacterial indicators of human faecal wastes were not available. As for coliform bacteria, they are widely distributed in both human and animal faeces thus making it difficult to be a reliable indicator of strictly human input.

As early as 1958, Mossel (1) proposed bifidobacteria as a potential indicator of faecal contamination. Cabelli (2), Resnick and Levin (3) suggested the same due to the fact that *Bifidobacterium longum* and *B. adolescentis* are the two most often and most abundant human faecal isolates found from raw sewage. Recent field study in Ontario by Seyfried *et al* (4) indicated concentrations of bifidobacteria were higher in sanitary and high priority storm sewage than in nonpriority storm water thus suggesting the ratio of bifidobacteria to *Streptococcus casseliflavus* counts may allow to trace sources of human and/or animal faecal wastes.

Commercially available bacterial detection systems may have a reported detection sensitivity of 1 CFU/100ml (5), but has a risk of false-positive or false-negative occurrences (6,7,8). Due to difficulty in monitoring bifidobacteria routinely by conventional culturing technique, as they tend to die off quickly after exposure to extra-intestinal environment, an alternate method has to be developed. Although Restriction Fragment Length Polymorphism (RFLP) analysis, using 23S rDNA (rRNA gene) as gene probe, allows ribosomal gene restriction patterning for identification of *Bifidobacterium* strains (25), it does not have a species-specific ribosomal gene restriction pattern. Similarly, by comparing partial sequence of 16S rRNA obtained by sequencing with universal 16S rRNA primers A and C (26), Yamamoto *et al* (27) designed and tested five *Bifidobacterium* species-specific oligonucleotide probe (ranging from 16 bases to 19 bases in length) for five *Bifidobacterium* species (*B. adolescentis, B. bifidum, B. breve, B. infantis*, and *B. longum*). Unfortunately, they have not investigated any possible *Bifidobacterium* genus-specific probe, nor have they tested the sensitivity of those *Bifidobacterium* species-specific oligonucleotide probes. Thus other genomic region has to be studied for designing *Bifidobacterium* genus-specific probe and more advanced molecular biology technique has to be applied in order to maximize the detection sensitivity. To-date, the most sensitive detection tool is Polymerase Chain Reaction (PCR), it can be used to amplify a specific DNA segment up to a million fold. It has been reported that with PCR amplification, it is possible to detect a gene of interest with a single cell (9). Oligonucleotide hybridization, on the other hand, provides sequence specific detection and can distinguish one nucleotide difference (10). Combining both techniques, it has been possible to detect bacteria in clinical specimens and environment with high sensitivity and specificity (11,12,13,14,15,16,24). We propose to use a similar approach in developing a sensitive and a *Bifidobacterium* genus-specific oligonucleotide probe for monitoring human faecal contamination in waters. Accordingly, five common *Bifidobacterium* species (*B. adolescentis, B. bifidum, B. breve, B. infantis,* and *B. longum*) of human origins were chosen for this study. We report here the results of the isolation and characterization of a *Bifidobacterium* genus-specific DNA probe; the design of two PCR primers (BFL5-1 and BFL5-2) and a *Bifidobacterium* genus-specific oligonucleotide probe (BFP23).

MATERIALS AND METHODS:

Isolation of chromosomal DNA. Bifidobacteria were cultured in MRS media supplemented with L-cysteine at 37°C under anaerobic condition. Cells were harvested by centrifugation, washed once with RSB buffer (10 mM Tris-HCl, pH7.4, 10 mM NaCl, and 25 mM EDTA), and immediately frozen in liquid nitrogen. The frozen cell pellets were pulverized with a pestle in a mortar partially filled with liquid nitrogen. The powered cell lysate was then dissolved in 10 ml RSB buffer plus 1% sodium dodecyl sulphate (SDS). Proteinase K was added to a final concentration of 1 mg/ml and the digestion was carried out at 37°C for at least 2 hours. The cell lysate was extracted with phenol/chloroform and dialysed extensively against TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). RNA was removed by treating dialysed lysate with 20ug/ml DNase-free RNase A, followed by protein digestion with 200 ug/ml proteinase K. Chromosomal DNA was ridded of protein by extraction with phenol/chloroform and dialysis against TE buffer.

Construction of genomic library of *Bifidobacterium*. Chromosomal DNA of bifidobacteria was partially digested with *Alu I* or *Mbo I* and the resulting restricted DNA fragments were resolved by agarose gel electrophoresis. DNA fragments of desired size were excised from the gel, purified by Geneclean (Bio101Co.), ligated to linearized and dephosphorylated pUC19 vector and subsequently transformed to *E.coli* JM103 competent cells.

Slot-blot hybridization. DNA was denatured in 0.25N NaOH for 15 min, chilled on ice, transferred to slots of the manifold and vacuum-blotted onto Genescreen Plus membrane (Dupont Inc.) previously wetted with distilled water. DNA-blotted membranes were prehybridized in a solution of 50% formamide, 1% SDS, 10% dextran sulphate, 50 mM Tris-HCl pH7.5, and 100ug/ml denatured salmon sperm DNA at 42°C for 1 hr. Denatured radioactively-labelled DNA probe was added to a final concentration of 5-10 ng/ml (0.5 - 1.0 x 10^6 cpm). Hybridization was carried out at 42°C for 12-18 hr. The membranes were washed with increased stringency and autoradiography was done at -70°C.

PCR amplification of L5 region in bifidobacteria. A 288 bp coding region of L5 ribosomal protein gene, was amplified by PCR, using primers BFL5-1 and BFL5-2 (Fig. 5).1ug of chromosomal DNA in a reaction mix of 100 ul 1x Taq buffer (Promega corp.), 2.5 units of Taq polymerase, 1.5 mM $MgCl_2$, 600 pmol of BFL5-1, 1200 pmol BFL5-2, 10 mmoles of dNTP,was subjected to 30 cycles of denaturation (94°C, 1 min. 20 sec.), annealing (62°C, 40 sec.), and extension (72°C, 40 sec.), followed by a final extension step of 72°C, 3 min.

Cloning of PCR product. PCR products were resolved by low-melting point agarose gel electrophoresis. DNA fragments of 288 bp were excised, purified by hot TE-saturated phenol extraction, ligated to *Eco*RV linearized, dephosphorylated pBluescript vector and subsequently transformed to *E.coli* DH5-alpha competent cells.

Sequencing of cloned PCR product. Two individual transformants from each of *B. adolescentis, B. breve* and *B. infantis* were sequenced with M13 reverse and universal forward primer using dideoxy sequencing method with Sequenase Version 2.0 DNA sequencing kit.

Southern transfer of PCR products. PCR products were resolved by 1.2% agarose gel electrophoresis and transferred onto Genescreen Plus membrane (Dupont Inc.) by vacuum transfer (Pharmacia Biotech Inc.) using 15 min. of depurination (2N HCl), 15 min. of denaturation (0.5M NaOH, 0.5M NaCl), 15 min. of neutralization (1M Tris pH 7.5, 1.5M NaCl), and 30 min. of high salt transfer (20xSSC) under constant vacuum strength of 55 cm H_2O.

Radioactive labelling of oligonucleotide using T4 polynucleotide kinase. *Bifidobacterium*-specific probe BFP23C (synthesized by Dalton Chemical Laboratories Inc.) was radioactively labelled in 20 ul of T4 polynucleotide kinase reaction containing 10 pmole BFP23C, 55pmole gamma ^{32}P-dATP (4500 Ci/mmole, ICN Biomedicals Canada Ltd.), 2 ul of 10xNEBuffer for T4 polynucleotide kinase, and 10 units of T4 polynucleotide kinase (New England Biolabs Inc.). Reaction was stopped by heating at 65^{0}C, 15 min. and radioactively labelled oligonucleotide was purified by passing through G-50 Sephadex spin-column.

Oligonucleotide hybridization. After Southern transfer, the air-dried membrane was prewetted in 2xSSC and prehybridized at 42^{0}C for 30 min. in 20 ml of prehybridization buffer containing 1mM EDTA pH8.3, 6xSSPE, 100mM phosphate buffer pH6.8, 0.5% SDS, 2mg denatured salmon sperm DNA. Radioactively labelled BFP23C probe was then added to a final concentration of 0.5 pmole/ml (1.5×10^{5}cpm/ml) and membrane was hybridized at 42^{0}C for four hours followed by room temperature 2xSSC wash for 15 min. and another 2xSSC wash at 62^{0}C for 30 min..Autoradiograph was done at -70^{0}C for three hours.

RESULTS AND DISCUSSION:

The main purpose of this project is to isolate DNA sequences that are conserved and unique to bifidobacteria which can be developed into *Bifidobacterium*-specific DNA probes.

We have constructed a genomic library of *B. adolescentis* in pUC19. One hundred and thirty recombinant clones were picked randomly from this library, their plasmid DNA were purified and hybridized to radioactively labelled chromosomal DNA of *B. longum*. These species were used because they are the most commonly found bifidobacteria in human faeces. It is reasoned that any clone which showed positive hybridization signal would contain a DNA insert that is conserved in both *B. adolescentis* and *B. longum.* Two *B. adolescentis* recombinant clones (pA34 and pA63) were found to show strong hybridization signals with *B. longum* DNA. These two clones did not cross-hybridize indicating they are from different segments of *B. adolescentis* DNA. Clone pA34 was characterized further and the results are presented.

Fig. 1 shows the results of slot-blot hybridization in which chromosomal DNA of *Bifidobacterium* and non-*Bifidobacterium* species were blotted onto nylon membrane and subsequently probed with radioactively labelled DNA of pA34. Probe pA34 hybridized strongly to DNA of *B. adolescentis* ATCC 15703, *B. longum* ATCC 15707, *B. breve* ATCC 15700 and *B. bifidum* ATCC 29521, but weakly to DNA of *Escherichia coli* and *Shigella soneii*, and not to DNA of *Lactobacillus casei*, *Campylobacter jejuni*, or *Pseudomonas aeruginosa*. These data demonstrate and confirm the high specificity of pA34 towards *Bifidobacterium* species.

The sensitivity of pA34 as DNA probe in detecting *Bifidobacterium* by radioactive means was then determined. Both DNA and cells of *B. adolescentis* were used in the hybridization analysis. Decreasing amounts of *B. adolescentis* DNA were slot-blotted onto nylon membrane followed by probing with radioactively labelled pA34. Hybridization signals were detected in slots containing as low as 10 ng of chromosomal DNA of *B. adolescentis* (Fig. 2). When *B. adolescentis* cells were used, the minimum number of cells that could be detected by pA34 was 10^5 cells. It is possible to enhance the sensitivity of hybridization by amplifying bifidobacteria DNA with PCR prior to hybridization analyses.

As mentioned above, pA34 is specific to at least four *Bifidobacterium* species but it also cross-hybridizes weakly to genomic DNA of *E. coli* and *S. soneii* suggesting part of pA34 shares some homology to *Escherichia* and *Shigella*. The specificity of pA34 to bifidobacteria can be enhanced by eliminating sequences not conserved in bifidobacteria, or conserved in other species of bacteria. We thus undertook the task of sequencing the DNA insert of pA34, which contains a DNA insert of 352 basepairs. Its DNA and translated protein sequences are depicted in Fig. 4. An open reading frame is found in the DNA sequence and it shows 64% homology to part of the DNA sequence (from nucleotides 144 to 495) of ribisomal protein L5 of *E. coli*. This explains the weak hybridization signal detected in genomic DNA of *E. coli* by pA34 in slot-blot hybridization. The translated sequence of pA34 is 59% homologous to L5 protein of *E. coli*. The high homology between the two sequences at the protein level suggests strongly that pA34 contains a portion of the ribosomal protein L5 gene of *B. adolescentis*. Neither start nor stop codons are found in pA34 indicating the sequence is incomplete. We expect the *B. adolescentis* L5 protein gene is of similar size to its 539 basepair counterpart in *E. coli*.

In order to evaluate the full potential of using the L5 gene in the development of a PCR assay for specific detection and identification of *Bifidobacterium*, we need to determine the degree of conservation of the L5 gene between species of *Bifidobacterium* and bacteria of different genera and families. To-date in the databank no L5 ribosomal protein gene sequence has been submitted for any species of *Bifidobacterium* but there are 20 submissions of L5 protein sequences for other eubacteria and archea bacteria that shows homology to the A34 polypeptide (117aa). This search was done recently using the BLAST program. The *Micrococcus luteus* L5 protein (a recent submission) showed the highest homology with 70% identity(data not shown). Eleven of the L5 gene sequences were retrieved from the databanks (Genbank) and they were aligned and compared using the CLUSTAL V program. Fig. 4 shows the alignment of the L5 (A34) nucleotide sequence of *B. adolescentis* with the top 7 most homologous L5 sequences of other eubacteria. Four fairly conserved regions can be observed but there are large regions that show low conservation. Two of the four conserved regions have been chosen to design two PCR degenerate primers (see Fig. 4). These two degenerate primers should amplify a DNA fragment of about 290 bp from the various species of *Bifidobacterium*, (*B. breve, B. longum, B. infantis and B. bifidum*). By optimizing the PCR reaction, an amplified fragment of about 290 bp was observed for all five bifidobacteria (see Fig. 6)

The PCR products of *B. adolescentis, B. breve* and *B. infantis* were successfully cloned into pBluescript. The DNA sequences of the cloned PCR fragments from *B. adolescentis, B.*

breve and *B. infantis* were aligned and showed high homology; 95% identity between *B. breve* and *B. infantis*, 92% identity between *B. adolescentis* and *B. infantis*, 91% identity between *B. adolescentis* and *B. breve* (see Fig. 7). The longest stretch of conserved DNA sequence among the existing three sequences lies between nucleotide 209 and 221. This conserved sequence of 23 nt (CTCACCGAGCAGTCCATGTTCC) is a potential candidate for development of a *bifidobacterium*-specific oligonucleotide probe. This region is aligned with the homologous sequence from seven other bacteria including *Thermus flavus* (17), *Escherichia coli* (18), *Bacillus subtilis* (19), *Cyanophor paradoxa* (20), *Chlamydia trachomatis* (21), *Mycoplasma capricolum* (22), and *Micrococcus luteus* (23). As can been seen in Fig. 8, none of the seven sequences showed 100% homology to the conserved bifidobacteria 23 nt sequence, with the highest match of 19 out of 23 for *M. luteus* and as low as 11 out of 23 for *C. trachomatis*.

We synthesized the complementary strand of BFP23 to determine its potential as a *Bifidobacterium*-specific oligonucleotide probe (BFP23C:5'-GGA ACA TGG ACT GCT CGG TGA G-3'). DNA samples from five of the *Bifidobacterium* species (*B. aldolescentis, B. breve, B. bifidum, B. infantis* and *B. longum*) and seven species of other bacteria (*Arcobacter nitrofigilis, Campylobacter jejuni, Escherichia coli,Helicobacter pylori, Lactobacillus caseii, Pseudomonas aeroginosa,* and *Shigella soneii*) were tested. Each bacterial DNA (1ug) was subjected to PCR amplification using primers BFL5-1 and BFL5-2, and 10ul out of the 100ul PCR reaction mixture from each sample was analysed in 1.2% agarose gel electrophoresis and DNA bands were stained with ethidium bromide (Fig. 9A). All five *Bifidobacterium* species,*B. alsolescentis* (Lane 3), *B. bifidum* (Lane 4), *B.breve* (Lane5), *B. infantis* (Lane 6), and *B. longum* (Lane 7) produced an intense band of 288bp. As for the seven other bacteria, *L. caseii* (Lane 12) showed a strong definite band at 288bp,*A. nitrofigilis* (Lane 8) did not show any definite PCR product, while the rest (Lane 9:*C. jejuni*, Lane 10:*E. coli*, Lane11:*H. pylori*, Lane13:*P. aeroginosa*, and Lane 14:*S. soneii*) showed weak bands at 288bp and 200bp. A strong diffuse band below 100bp in all lanes was probably due to primer-dimer by-products of unused primers. The 288bp band observed in six non-Bifido bacteria (*C. jejuni, E.coli, H. pylori, L. caseii, P. aeroginosa,* and *S. soneii*) presumably was produced because BFL5-1 and BFL5-2 were designed from two conserved regions of the L5 ribosomal protein nucleotide sequence of eight diverse bacteria.

Due to the high degeneracy of primers BFL5-1 and BFL5-2, PCR products of the right size do not indicate amplification of the right targeted gene region, futher confirmation is needed. The DNA bands in the agarose gel show in Fig. 9A were transfered onto a Genescreen Plus membrane(Dupont Inc.) and hybridized with the 5' end-labeled BFP23C probe. Although the theoretical melting temperate (Tm) is 64°C, the experimental Tm was determined emperically by washing the hybridized membrane for 30 min. with 2xSSC at room temperate, 50°C, 60°C, 62°C, and 65°C successively. Up until 62°C, all five *Bifidobacterium* showed strong hybridization signal at 288bp (Fig. 10B, only results of 62°C are shown). With the exception of *P. aeroginosa* and *S. soneii*, which showed weak hybridization signal at 288bp, no hybridization signal was observed for the other bacteria. At 65°C, hybridization probe in *B. bifidum* and *S, soneii* got washed off, while *B. aldolescentis, B. breve, B. infantis,* and *B. longum* retained their hybridization signals. However, *P. aeroginosa* also retained its weak hybridization signal (Data not shown). These results indicate that the 23-nt BFP23 region is not completely conseved in all five *Bifidobacterium* species. Even at 65°C, the weak hybridization band of *P. aeroginosa* persisted and considering the 288bp fragment was not PCR amplified to a high level (see Fig. 9A), the L5 ribosomal protein sequence at the BFP23 region in *P. aeroginosa* must be very similar to that of *B. aldolescentis, B. breve, B. infantis,* and *B. longum.*

To determine the sensitivity of the detection system using PCR primers BFL5-1 and BFL5-2 and labeled BFP23C, serially diluted *B. bifidum* DNA was subjected to BFL5-1 and BFL5-2 PCR reaction . The amount of *B. bifidum* DNA used in the PCR reaction (100ul) is as follow: Lane 3(100ng), Lane 4(10ng), Lane 5(1ng), Lane 6(100pg), Lane 7(10pg), Lane 8(1pg), Lane 9(100fg), Lane 10(10fg), Lane 11(1fg), and Lane 12(0.1fg). 10ul from each each of the PCR reactions was analysed in 1.2% agarose gel electrophoresis(Fig. 10A). When such ethidium bromide stained gel was viewed under UV transillumination, an expected 288bp band showed strongest intensity in Lane 3(100ng) and the band was barely detectable in Lane 8(1pg) and dissappeared in Lane 9(100fg). In order to confirm the identity of the 288bp bands, DNA was transfered onto Genescreen Plus membrane(Dupont Inc.) and hybridized with radiolabeled BFP23C, washed at 62°C in 2xSSC for 30 min.

Autoradiograms were developed after four hours and hybridization signals at the 288bp position were observed from Lane 3 (100ng) down to Lane 8 (1pg). Assuming the *Bifidobacterium* genome size is about the size of the *E.coli* genome, $3x10^9$ daltons(28), the assay could detect as low as 200 copies of *Bifidobacterium* genome. However, only 10ul out of the total 100ul of PCR products was used, the actual amount of detectable signal is 20 copies.

CONCLUSION:

This study proved BFL5-1 and BFL5-2 can amplify specific segment of L5 ribosomal protein gene in the five chosen *Bifidobacterium* (*B. aldolescent, B. bifidum, B. breve, B. infantis,* and *B. longum*). BFP23C hybridized strongly to all five chosen *Bifidobacterium* at 62°C, weakly to *P. aeroginosa* , and negative to the other six targeted bacteria. The detection limit of this assay is 20 copies of the targeted gene. The detection assay can be improved by decreasing the degeneracy of the PCR primers and design an oligonucleotide probe in another conserved region of the L5 gene sequence for the five *Bifidobacterium* species. d.

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FIGURES

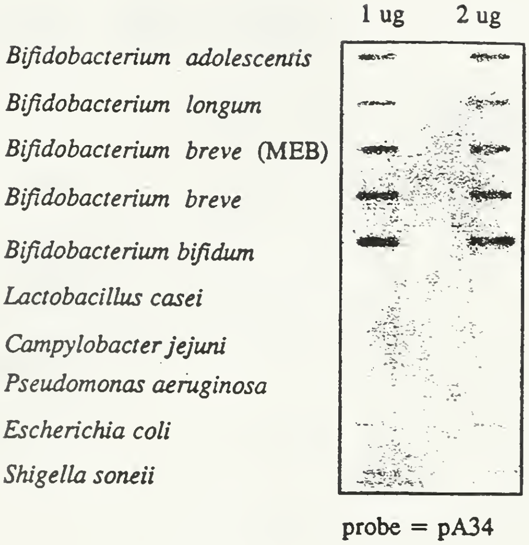

Figure 1. Slot-blot hybridization of genomic DNA of various bifidobacteria and non-bifidobacteria to DNA probe pA34.

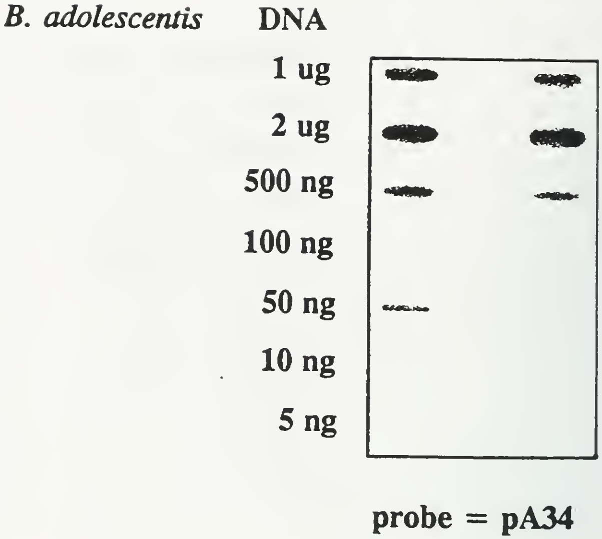

Figure 2. Determination of sensitivity of DNA probe pA34. Decreasing amounts of *B. adolescentis* DNA slot-blotted onto nylon membrane were hybridized to radioactively-labelled pA34.

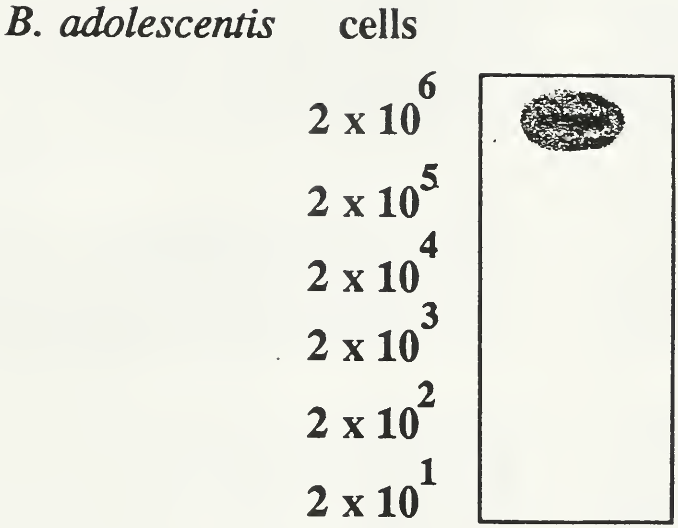

Figure 3. Determination of sensitivity of DNA probe pA34. Decreasing numbers of *B. adolescentis* cells were lysed and slot-blotted onto nylon membrane followed by hybridization to radioactively-labelled pA34.

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CLUSTAL V multiple sequence alignment

Bifidobact    CTCATCGAAGGCGCTGTCAA-GGACCTCACTCTGATCACCGGCCAGAAGC
Thermus       ATCCTGGAGAAGGCGGCCCA-GGAGCTCGCCCTCATCACGGGCCAGAAGC
E. coli       CTGCTGGATAACGCAGCAGC-AGACCTGGCAGCAATCTCCGGTCAAAAAC
Bacillus      GCAATCGACAGTGCTGTTGA-GGAATTAACGTTTATCGCAGGTCAAAAAC
 Cyanophor    ATTTTAGAAGCTTCTGTTAAAGAAATTACTGAAATTACC-GGTCAAAAAG
Chlamydia     CTTTTCCAGGCTCAT-TTAGAGGAATTGGCGGTTATCTCTGGTCAAAAAC
Mycoplasma    AAATTAGATGCAGCTAT-ATTTGAATTAGAAAAATTGTCTGGACAAAAAC
Micrococcu    ATCATCGACGACGCCGTCAC-CGACCTCACCGCCATCACCGGCCAGAAGC
                  * .*               *  *        *  * ** ** **

              →
Bifidobact    CGAAGATCACCAAGGCTAAGAAGTCTGTCGCCGCA-GTTCCACCTGCCTGA
Thermus       CCGCGGTCACCCGGGCCAAGAAGTCCATCTCCAA-CTTCAAGCTCCGCAA
E. coli       CGTTGATCACCAAAGCACGCAAATCTGTTGCAGG-CTTCAAAATCCGTCA
Bacillus      CTGTCGTTACTCGTGCCAAGAAATCAATTGCTGG-ATTCCGTCTTCGTGA
 Cyanophor    CTATTGTTACTAGAGCCAAAAAAGCTATTGCTGGT-TTTAAATTAAGACA
Chlamydia     CTTTGGTAACAAGAGCTAAAAACTCTATCGCAGGC-TTCAAGTTACGAGA
Mycoplasma    CAATTGTTACAAAAGCTAAAAAA-TCATTAGCAGTATTTAAGTTAAGAGA
Micrococcu    CGATGATCACCAAGGCCCGCAAGTCCATCGCCGCA-GTTCAAGCTGCGTGA
              *     * **    **    **     *        **     *  *  *

Bifidobact    GGGCCAGGCCATCGGCGCCTACGTCACCCTGCGTGGCGAGCGCATGTGGG
Thermus       GGGGATGCCCATCGGCCTCGGGGTCACCCTGCGGCGTGACCGGATGTGGA
E. coli       GGGCTATCCGATCGGCTGTAAAGTAACTCTGCGTGGCGAACGCATGTGGG
Bacillus      GGGAATGCCTATCGGTGCGAAAGTAACTCTTCGCGGAGAGCGCATGTATG
 Cyanophor    AGATATGCCTATTGGTGTTATGGTTACTTTACGCGGTGATTATATGTATG
Chlamydia     GGGTCAGGGCATCGGAGCAAAAGTCACTCTACGTGGAATCCGTATGTATG
Mycoplasma    AGGTATGGCAATTGGTGCTAAAGTTACTTTAAGAGGTAAAAAAATGTATG
Micrococcu    GGGCATGCCCATCGGCACGCACGCCACCCTCCGTGGCGATCGCATGTGGG
               *        ** **       *  **  *  *  *       ****

Bifidobact    AGTTCCTGGATCGCCTTCTGACCATGGCTCTGCCGCCTATCCGCGATTTC
Thermus       TCTTCCTGGAAAAGCTCCTCAACGTGGCCCTTCCCCGCATCCGCGACTTC
E. coli       AGTTCTTTGAGCGCCTGATCACTATTGCTGTACCTCGTATCCGTGACTTC
Bacillus      ATTTCCTTGATAAACTTATTTCTGTATCTTTACCGCGTGTACGTGACTTC
 Cyanophor    CTTTCTTGGATCGTTTAATTAATTTATCTTTACCAAGAATTCGAGATTTT
Chlamydia     ACTTTATGGACCGTTTTTGCAATATTGTCTCCCCAAGAATTCGAGACTTT
Mycoplasma    ACTTTTTAGATAAGTTAATTAATGTTGCTTTACCAAGAGTACGTGACTTT
Micrococcu    AGTTCCTGGACCGCCTGGTCACGCTGCCGCTGCCGCGCATCCGTGACTTC
                **  * **     *        *       **  *  * ** ** **

Bifidobact    CGCGGCATCAACGGCGACCAGTTCGATGGTCAGGGCAACTACAACTTTGG
Thermus       CGGGGGCTCAACCCCAACAGCTTTGACGGCCGGGGCAACTACAACCTGGG
E. coli       CGTGGCCTGTCCGCTAAGTCTTTCGACGGTCGTGGTAACTACAGCATGGG
Bacillus      CGCGGGGTTTCTAAAAAATCTTTCGACGGTCGCGGTAACTACACACTTGG
 Cyanophor    CGCGGTATTACTGCAAAAAGTTTTGATGGTCGTGGAAATTATAATCTTGG
Chlamydia     AGAGGATTCTCTTGTAAAGGA---GATGGACGAGGATGTTATTCCCTTGG
Mycoplasma    AGAGGAGTTTCAAAAACTTCATTTGATGGATTTGGAAATTTTTACACTGG
Micrococcu    CGCGGCCTGTCCGACCGCCAGTTCGACGGCAACGGCAACTACACCTTCGG
               * **  *                ** **    **    *        **

Bifidobact    CCTCACCGAGCAGTCCATGTTCCACGAGATCGATCCCGATTCGATCGATC
Thermus       CCTGAGGGAGCAGCTCATCTTCCCCGAGATCACCTACGACATGGTGGACC
E. coli       TGTCCGTGAGCAGATCATCTTCCCAGAAATCGACTACGATAAAGTCGACC
Bacillus      TATCAAAGAACAGTTAATCTTCCCTGAAATTGACTACGATAAAGTAACAA
 Cyanophor    TTTAAAAGAACAATTGATTTTTCCAGAAGTTGATTATGATAGTATTGAAC
Chlamydia     TTTAGATGATCAGCAAATCTTTCCTGAAGTTGATTTAGATGCTGTTAAAC
Mycoplasma    AATTAAAGAACAAATTATTTTTCCAGAAGTAGATCATGATAAAGTT-ATT
Micrococcu    CCTGTCCGAGCAGACCGTGTTCCACGAGATCGATCAGGACAAGATCGACC
                *    ** **     * ** *  **  *       **     *
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                      <-----------------
Bifidobact      ACCAG-CGTGGTATGGACATCACCGTGGTGACCAGCACCAAGGACGACAA
Thermus         CCCTG-CGGGGCATGGACATCGCGGTGGTGACCACCGCCGAGACCGACGA
E. coli         GCGTT-CGTGGTTTGGACATTACCATTACCACTACTGCGAAATCTGACGA
Bacillus        AGGTT-CGCGGAATGGACATCGTTATCGTAACAACTGCTAATACTGACGA
 Cyanophor      AAATT-CGCGGTATGGATATTTCAATTGTAACCACTGCAAAAACAAATCA
Chlamydia       -GATCTCAGGGAATGAATATTACTTGGGTAACTACAGCACAAACCGATGC
Mycoplasma      AGATTACGTGGTATGGATATTACTATTGTAACTTCTGCTAAAACAAATAA
Micrococcu      GCGTG-CGCGGCATGGACATCACCGTGGTGACGACCGCCAAGAACGACGA
                      *  **  ** * **           **    *  *     *

Bifidobact      GGAAG---
Thermus         GGAGG---
E. coli         AGAAG---
Bacillus        AGAAG---
 Cyanophor      AGAAG---
Chlamydia       GGAGTGCC
Mycoplasma      AGAAG---
Micrococcu      CGAGG---
                 **
```

B. adolescentis		ATC	ACC	GGC	CAG	AAG	CCG	
		Ile	Thr	Gly	Gln	Lys	Pro	
BF L5-1	5′	ATC	ACC/G	GGG/T/C	CAG	AAG	CCC/G	3′

B. adolescentis		CGT	GGT	ATG	GAC	ATC	ACC	
		Arg	Gly	Met	Asp	Ile	Thr	
BF L5-2	3′	CN	CCC/G/A	TAC	CTG	TAG	TGG/C	5′

Fig. 5 Based on the translated amino acid sequence from Fig. 4, two degenerate oligonucleotides were designed for L5 ribosomal gene amplification, namely BF L5-1 (5′ primer) and BF L5-2 (3′ primer).

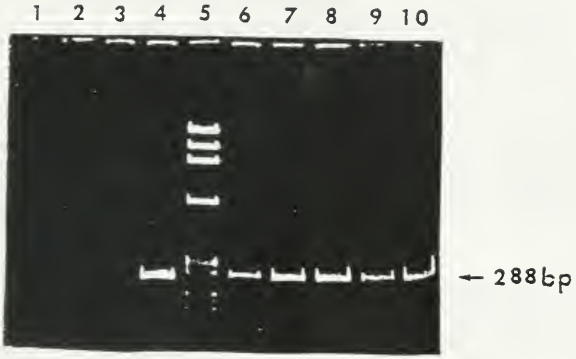

Fig. 6 PCR amplification of L5 encoding fragment from various species of *Bifidobacterium*. PCR amplification was done using 1 ug DNA (if added), 10 mmoles of dNTP, 600 pmoles primer BFL5-1, 1200 pmoles primer BF L5-2, 0.25 unit of Tag polymerase, 1.5 mM $MgCl_2$ in a total volume of 100 ul. Thirty cycles of denaturation (94°C, 1 min. 20 sec.), annealing (60°C, 40 sec.), and extension (72°C, 40 sec.) were used, and followed by a final extension of 72°C, 3 min. 10 ul of PCR products were analyzed by 6% polyacrylamide gel electrophoresis. Lanes 1-3 are negative control, lane 1 (no DNA), lane 2 (*Campylobacter jejuni* DNA), lane 3 (pUC19), lane 4 (pA34), lane 5 (0.5 ug molecular weight markers Ø x 174 DNA cut with *Hae* III), lane 6 (*B. adolescentis*), lane 7 (*B. bifidum*), lane 8 (*B. breve*), lane 9 (*B. infantis*) and lane 10 (*B. longum*).

```
                1
B. adolescentis AAGATCACCA AGGCTAAGAA GTCTGTCGCG CAGTTCCACC TGCGTGAGGG
B. breve        AAGATCACCA AGGCCAAGAA GTCCGTTGCG CAGTTCCACC TGCGAGAAGG
B. infantis     AAGATCACCA AGGCCAAGAA GTCCGTCGCG CAGTTCCACC TGCGTGAGGG
                ********** **** ***** *** ** *** ********** **** ** **

                51
B. adolescentis CCAGGCCATC GGCGCCTACG TCACCCTGCG TGGCGAGCGC ATGTGGAGT
B. breve        CCAGGCCATC GGTGCATACG TCACCCTGCG CGGCGACCGT ATGTGGAGT
B. infantis     CCAGGCCATC GGTGCGTACG TCACCCTGCG TGGCGACCGC ATGTGGAGT
                ********** ** ** **** **********  ***** **  **********

                101
B. adolescentis TCCTGGATCG CCTTCTGACC ATGGCTCTGC CGCGTATCCG CGATTTCCGC
B. breve        TCCTGGATCG TCTGCTGACC CTTGCGCTGC CCCGTATCCG CGACTTCCGC
B. infantis     TCCTGGACCG TCTGCTGACC CTCGCCCTGC CCCGTATCCG CGACTTCCGC
                ******* **  ** ******  * ** **** * ******** *** ******

                151
B. adolescentis GGCATCAACG GCGACCAGTT CGATGGTCAG GGCAACTACA ACTTTGGCCT
B. breve        GGTATCAACG GTCACCAGTT CGATGGCCAG GGCAAGTACA ACTTTGGTCT
B. infantis     GGCATCAACG GCCACCAGTT CGATGGCCAG GGCAACTACA ACTTTGGTCT
                ** ******* *  ******* ****** *** ***** **** ******* **

                201
B. adolescentis CACCGAGCAG TCCATGTTCC ACGAGATCGA TCCGGATTCG ATCGATCACC
B. breve        CACCGAGCAG TCCATGTTCC ATGAGATCGA TCCTGATTCG ATCGATCACG
B. infantis     CACCGAGCAG TCCATGTTCC ACGAGATCGA TCCTGATTCG ATCGATCACG
                ********** ********** * ******** *** ****** *********

                251
B. adolescentis AGC
B. breve        TGC
B. infantis     TGC
                 **
```

Fig. 7 Alignment of PCR fragment sequence without BFL5-1 and BFL5-2 sequences. Underlined is the proposed *Bifidobacterium*-specific oligonucleotide (BFP 23). "*" indicates conserved nucleotides.

Bifidobacterium	CTCACCGAGCAGTCCATGTTCCA	# of matches
Thermus	CTgAggGAGCAGctCATcTTCCc	16/23
E. coli	gTCcgtGAGCAGatCATcTTCCc	15/23
Bacillus	aTCAaaGAaCAGTtaATcTTCCc	15/23
Cyanophor	tTaAaaGAaCAaTtgATtTTtCc	12/23
Chlamydia	tTagatGAtCAGcaaATcTTtCc	11/23
Mycoplasma	aTtAaaGAaCAaattATtTTCCc	12/23
Micrococcus	CTgtCCGAGCAGaCCgTGTTCCA	19/23

Fig. 8. Alignment of proposed *Bifidobacterium*-specific oligonucletide with seven other bacteria including *Escherichia coli*, *Bacillus subtilis*, *Cyanophor paradoxa*, *Chlamydia trachomatis*, *Mycoplasma capricolum*, *Micrococcus luteus*, and *Thermus flavus*.

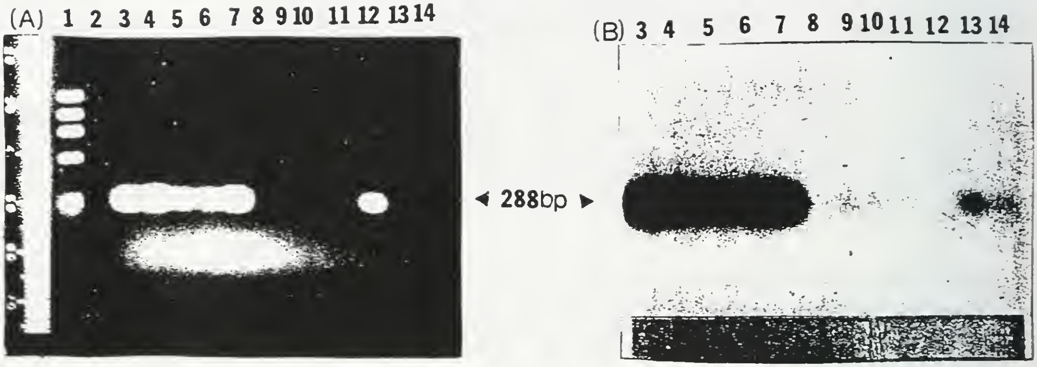
(A) 1 2 3 4 5 6 7 8 9 10 11 12 13 14
(B) 3 4 5 6 7 8 9 10 11 12 13 14
288bp

Fig. 9 PCR amplification with primers BFL5-1 and BFL5-2 and Southern blot hybridization with probe BFP23C. DNA from five *Bifidobacterium* species and seven other bacteria were amplified using primers BFL5-1 and BFL5-2. The PCR products were analysed by 1.2% agarose gel electrophoresis (A). The gel was transfered onto Genescreen Plus membrane and probed with radiolabeled BFP23C (B). In both A and B, Lanes: 1, phi-X 174 *Hae III* marker; 2, no DNA control; 3, *Bifidobacterium aldolescentis*; 4, *B. bifidum*; 5, *B. breve*; 6, *B. infantis*; 7, *B. longum*; 8, *Arcobacter nitrofigilis*; 9, *Campylobacter jejuni*; 10, *Escherichia coli*; 11, *Helicobacter pylori*; 12, *Lactobacillus caseii*; 13, *Pseudomonas aeroginosa*; 14, *Shigella soneii*.

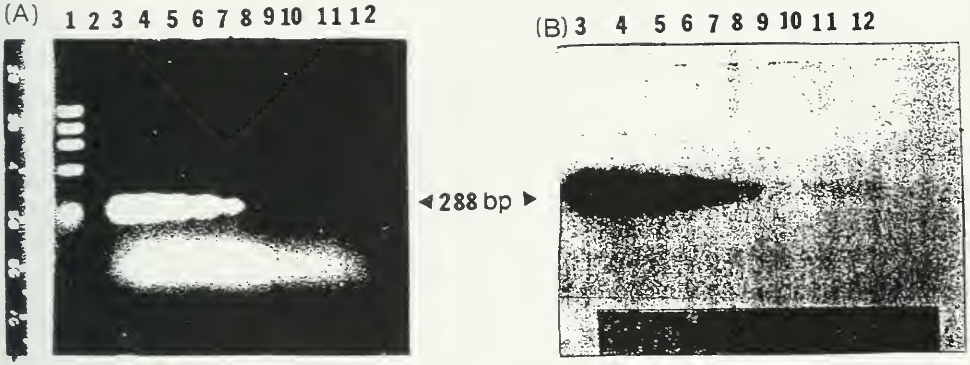

Fig. 10 Sensitivity of detection of *B. bifidum* DNA with probe BFP23C. Serially diluted *Bifidobacterium bifidum* DNA was subjected to BFL5-1 and BFL5-2 PCR amplification, then analysed by 1.2% agarose gel electrophoresis (A), followed by Southern blot hybridization with radiolabeled BFP23C oligonucleotide probe (B). In both A and B, Lanes: 1, phi-X 174 *Hae III* marker; 2, no DNA control; 3, 100ng of *B. bifidum* DNA; 4, 10ng; 5, 1ng; 6, 100pg; 7, 10pg; 8, 1pg; 9, 100fg; 10, 10fg; 11, 1fg; and 12, 1ag.